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<u>Subject: Staining-Bacterial Cell Structure</u> <u>Lecture Number: 3</u> <u>Done by: Joud Baki</u> <u>Corrected by: Issa Deir</u>

Principles of staining:

- <u>Revision:</u>

- Stains can be either simple or differential
- Gram stains are mainly used as differential stains.
- Outputs of gram staining:
 - <u>Gram positive</u>: Have a thick cell wall.
 - Gram negative: Don't retain the stain and are stained pink.
 - Gram variable: Uneven staining.
 - <u>Gram non-reactive</u>: Bacteria that don't take any of the two stains, and they remain unstained (eg: mycobacteria)

• Mycobacteria:

An important genus of the bacteria that causes tuberculosis (TB) and leprosy. It has a thick cell wall that looks like the cell wall of a gram positive bacteria. This cell wall is not <u>only</u> made up of peptidoglycans (as in gram positive), but is also rich in a certain substance called *mycolic acid* which is highly *hydrophobic* (a wax-like material) to resist staining (prevent stain passage in or out of the cell) in general under normal circumstances. This is why a special stain is used for mycobacteria which is the <u>Ziehl-Neelsen</u> <u>(acid-fast) stain.</u> It is the most important *differential* stain used, especially for identification of bacteria. This stain also depends on the cell wall structure. Certain procedures are done to stain and detect mycobacteria, which are similar to gram staining with minor differences.

Procedures:

1. Carbolfuchsin stain is added to the sample.

Carbolfuchsin is a cationic red dye. It has a high affinity to other bacterial components. If we have a sample containing mycobacteria and any other bacteria (gram positive bacteria for example), carbolfuchsin will stain any bacteria present in the sample except for mycobacteria due to its

hydrophobic cell wall which prevents stains to enter (especially charged stains). Note: Most stains are positively charged (hydrophilic).

2. Heat and rinse the sample.

Heating is used to force staining. It increases the permeability of the hydrophobic cell wall of mycobacteria, so the stain will be enforced inside the cell. Before heating, gram positive and gram negative bacteria were stained, while after heating, all the bacteria present (including mycobacteria) will be stained.

3. Decolorize with 3% HCl in ethanol and rinse

Since all bacteria are stained, our aim now is to differentiate between different types of bacteria. Decolorization is done by using ethanol or ethanol/acetone followed by rinsing or washing with water (the same way as in gram staining). Gram negative bacteria will be decolorized, While gram positive bacteria will be stained and will not be decolorized This is why 3% of HCl is used. This strong decolorizing agent (HCl with ethanol) will decolorize both gram positive and gram negative bacteria. Mycobacteria will not be decolorized <u>even with a strong decolorizing agent</u>, because when heating is removed, the hydrophobic cell wall will become impermeable to stains again (prevent stain passage), so the stain will not exit the cell and will stay stained. So after this step, mycobacteria will remain red, while the other bacteria will become colorless.

4. Counter-stain with methylene blue

In the previous steps, we managed to differentiate mycobacteria from other bacteria. Now, in order to visualize colorless bacteria, a counter-stain called methylene blue is used, which gives a blue color. The blue color of methylene blue can't overcome the red color of mycobacteria, because there is no heating in this process, so the methylene blue stain can't enter the cell, and this is the major difference between the initial staining and counter-staining (in initial staining, carbolfuchsin can enter the cell since heat is applied, while in counter-staining, methylene blue can't enter the cell since there is no heat). Then, the sample is put under the microscope, and we will see that mycobacteria have been stained red, while the other bacteria (gram positive and gram negative bacteria) have been stained blue. In other words, most bacteria will **lose** the red stain when decolorized & will be stained with blue counterstain, WHILE *acid fast bacteria* (i.e. mycobacteria) will **retain** the red color.

• Special staining:

Certain stains that are used to identify specific structures of bacterial cells. We have a single type of bacteria (pure culture), and our aim is to visualize or identify a certain structure in this bacterial cell. On the other hand, differential stains are used to differentiate between different types of bacteria, not to identify special structures.

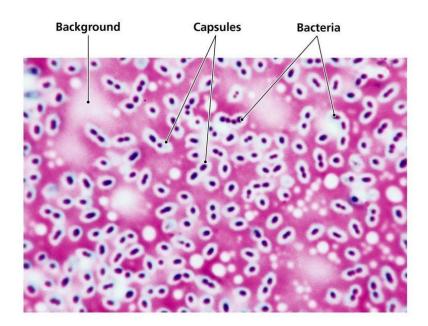
1. Negative staining

In order for any structure to be stained, it must have a high affinity to the stain used. When we want to visualize a certain structure that has a low affinity to a stain (resist taking up the stain), we stain everything except this structure, and we can differentiate it due to the contrast obtained (the structure is colorless while everything else is stained). This is called negative staining.

One of the most important structures following negative staining is **capsule.** When bacteria infect the human body, the most important defense line that is used by the body to attack bacteria is the immune system. The immune system is highly specific, which means that no immune reaction will occur unless antigens are recognized. In order for the immune system to detect the bacteria as foreign bodies, it must recognize the antigens of these bacteria. Some bacteria try to escape from the attack from the immune system by secreting an abundant amount of certain polysaccharides that coats the bacteria, so the immune system will not recognize the antigen since it is coated. This protective polysaccharide layer is called the capsule. So a capsule covers up the antigens, but it is not a permeability barrier.

The capsule is considered a <u>virulence factor</u> in the bacteria. Virulence factors are the weapons bacteria use to attack our body or to evade the attack from our immune system. This means that it is important to see whether a bacteria forms a capsule or not, because if it does, more aggressive treatment is needed since the contribution of the immune system will decrease. As the number of virulence factors in a bacteria increases, its pathogens will increase; this is why it is important to detect the virulence factors in the bacteria.

The problem in the capsule is that it has no charge (since polysaccharides are uncharged), so it can't be stained with an anionic or a cationic dye. If we stain a bacteria that forms a capsule with crystal violet (or crystal blue) stain, the cell will be stained but the capsule won't. Under the microscope, the cell will appear colored, but we can't identify whether it is coated with capsule or not since the capsule and the background are colorless. To solve this problem, we stain the background with an anionic dye (usually **acidic** such as nigrosin) to obtain a dark (red) background in order to form a contrast. If the bacteria is coated with capsule, a colorless layer will be easily seen between the cell and the background since the size of the capsule is very large (larger than the cell itself!). If the bacteria is not coated with capsule, no colorless layer will be seen.



2. Flagellar staining:

Some bacteria such as bacillus bacteria have flagella. Flagella(السياط) are appendages(زوائد) that some bacterial cells have and use for locomotion (التحرك). They are considered virulence factors in many cases (like capsules). They are very long BUT ALSO very thin to be seen easily with the light microscope, even though it can be stained with crystal violet. (Its thickness is less than the diffraction limit of a light microscope, which is 200 nm).

In order to see the flagella under the light microscope, we must increase its thickness by building up layers of stain on its surface. This is done by staining the flagella with a cationic (+ve) dye at first forming a mono-layer, then we stain it with an anionic dye (-ve) forming another mono-layer, then a cationic dye, then an anionic dye and so on. This process of coating layer by layer will result in accumulation of multi-layer stains leading to a larger thickness of flagella which will then be seen under the light microscope.

3. Endospore staining (Schaeffer-Fulton spore stain)

Spores (أبواغ) are produced by bacteria and fungi.

- In fungi they are called <u>exospores</u>. Used for reproduction
- In bacteria, they are called <u>endospores</u>. Used for survival

Bacteria produce spores to resist very harsh conditions (such as antibiotics, disinfectants and toxic chemicals). In order to survive these conditions, bacterial cell will replicate, but instead of having 2 daughter cells, they form only one cell that contains a copy of its DNA, and spores are formed around the formed DNA. Barrier properties for endospores are very high. They will protect DNA by preventing the entry of almost anything including harsh conditions (even water).

When these harsh conditions are gone, the bacteria (eg: bacillus) will return to normal conditions and will transform into another form of bacterial life called <u>vegetative cell</u>. The difference between the vegetative cells and endospores is that endospores stop all metabolic activities and resist penetration of anything inside the bacteria including dyes, so they appear as clear areas within bacterial cells, while vegetative cells can be stained. This is why if we stain a bacteria that forms endospores, vegetative cells will be stained while endospores won't, since dyes can't penetrate the multiple layers of endospores. To stain endospores, heat must be applied to enforce the dye in (like mycobacteria). A special type of staining is used called <u>Schaeffer-Fulton spore stain</u> which can make spores easily visualized.

Procedures:

1. Heat-fixed smear is covered with malachite green.

Malachite green is a green anionic dye. We add this dye to a sample containing endospores and vegetative cells. The dye will stain the vegetative cells, but won't stain the endospores.

2. <u>Heated until steaming</u> (تبخر)

Heating is done to increase the spore's permeability to the dye (like in mycobacteria, but heating in endospores is much more excessive than heat applied in mycobacteria, because the permeability of endospores is much less than permeability of mycobacteria.) Heating must NOT reach boiling point, since vegetative cells are destroyed at this temperature.

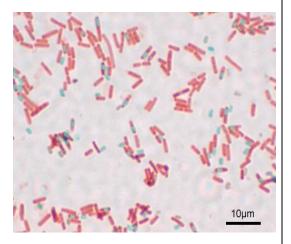
3. Rinse with water.

With a vegetative cell: Rinsing will decolorize it
With a gram positive bacteria: Rinsing will decolorize it since no mordant has been used.
With endospores: The dye will stay inside

So after this step, endospores will remain green, while the other bacteria will become colorless.

4. Counterstain with safranine (red).

Vegetative cells appear red, spores appear green.



Prokaryotes vs. Eukaryotes

- Living cells are classified into:

- 1. Prokaryotic cells (eg: Bacteria and Archaea)
- 2. Eukaryotic cells (all other cell types)

Differences:

1. The **determining criteria** between these two groups of cell types is the nucleus. Eukaryotic cells have a **TRUE** nucleus (presence of a nuclear envelope) whereas prokaryotic cells <u>DON'T</u>. What actually exists in prokaryotes is the nucleoid, which is the area that contains the genetic material DNA but is not surrounded by a nuclear envelope (membrane).

2. Prokaryotic cells are much **smaller** than eukaryotic cells because they have a simpler structure

3. Prokaryotic cells <u>DON'T</u> have **membrane-bound** organelles, such as mitochondria, golgi apparatus and endoplasmic reticulum, but eukaryotic cells <u>DO</u>. Ribosomes can actually be seen in prokaryotes because they are not membrane-bound organelles, and they are usually found in big numbers due to the high metabolic activity in these cells.

4. Prokaryotes contain a single <u>circular</u> chromosome (DNA) where both ends are attached to each other, but there are some exceptions where prokaryotes contain two chromosomes, whereas DNA in eukaryotes is <u>linear</u> in the form of paired chromosomes and they may reach up to 46 chromosomes as in human cells.

5. Prokaryotes reproduce by <u>binary fission</u> الانشطار الثنائي, while eukaryotes reproduce by mitosis and/or meiosis

TO SUMMARIZE:

Prokaryotes	Eukaryotes
DNA in circular chromosome, in nuclear region (nucleoid) not surrounded by membrane	DNA in paired chromosomes, in nucleus surrounded by membrane nuclear envelope
Lack organelles that are membrane enclosed	Organelles surrounded by membranes
Reproduction by binary fission	Mitosis and/or meiosis

Similarities: Both of them are living cells. What defines a living cell in general is that:

- 1. Both of them contain nucleic acid(s) (genetic material), whether it was DNA, RNA or both.
- 2. Both of them are surrounded by plasma membrane, which defines the boundaries of a living cell.
- 3. Both of them contain enzymes for metabolic activity.

20:00-26:00

• <u>The morphology of bacteria: size, shape and</u> <u>arrangement of prokaryotes</u>

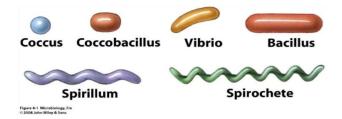
1. Size of bacterial cells

Since bacterial cells are prokaryotic cells, they don't contain membranebound organelles (as we mentioned before), which decreases their size making them much smaller than eukaryotes. Most bacterial cells are 0.5-2.0 μ m (or 0.5-5.0 μ m) in diameter, so we can see them under the light microscope. But there are exceptions, since bacteria is a very diverse group of, such as *Mycoplasma (0.3* μ m)-*Cyanobacteria (60* μ m, larger that most human cells) - *Thiomargarita (750* μ m, recently discovered in Africa and can be seen by naked eye as a dot). Bacteria is very abundant on earth, where the mass of bacteria on earth exceeds the mass of all the plant kingdom.

2. Shape of bacterial cells

A bacteria's cell wall gives every kind of bacteria a characteristic cell shape.

- Major shapes of bacterial cells are:
- a. <u>Spherical</u>: we call it <u>coccus</u>(plural is cocci).
- b. <u>Rod-shaped</u>: we call it <u>bacillus</u> (plural is bacilli).
 - Some bacteria are considered <u>coccobacilli</u> (between spherical shape and rod-shape). It is not considered a major shape.
- c. Spiral:
 - 1) Vibrio: Comma-shaped
 - 2) Spirillum: Rigid wavy-shaped
 - 3) Spirochete: Corkscrew-shaped, looks like a spring



✤ Other atypical shapes:

- مغزلي Spindle shape (1
- 2) Square
- مفصيص Lobed (3
- 4) Triangular

3. Arrangement of bacterial cells:

All prokaryotic cells are <u>single-cell organisms</u> (unicellular), but that doesn't mean they live separately. If we put a sample of bacteria under the microscope, are we going to see completely separated bacterial cells? Actually NO. We will notice that a group of bacterial cells will be intact to each other forming a characteristic arrangement, so that each type of bacteria is arranged in a specific way that is different from the other types.

This special arrangement is determined by the shape of the bacteria. For:

- <u>Spiral</u>: They don't form a special arrangement and each cell remains separated.
- <u>Bacilli</u>: They don't have a characteristic name. They form a chain-like arrangement, whether:
 - End to end
 - Side to side
- <u>Cocci</u>: They form many types of characteristic arrangements.

What causes this characteristic arrangement?

As we know, the bacterial cell reproduces by binary fission; the process in which the bacterial cell replicates its genetic material and its cell components, then forms a transverse septum in the middle of the cell which will eventually give two equal daughter cells.

So these characteristic arrangements of bacterial cells are due to cell division but without full separation (bacterial cells are not fully separated).

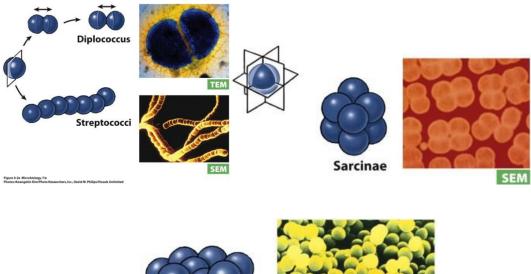
<u>Can we see individual (free) bacterial cells or are they always arranged</u> together?

Simply, we can find BOTH (for example we can find an individual rodshaped bacteria (bacillus) or chain like structures of bacilli).

As for the cocci, they are many arrangements:

- Division in 1 plane \rightarrow diplo (pair): Division without separation on one plane (every pair remain with each other)

- Division in 2 planes \rightarrow tetrads (4 cells in a cube)
- Division in 3 planes \rightarrow sarcinae (8 cells in a cube)
- Continuous division in 1 plane \rightarrow strepto (chain-like structure)
- Random division (no defined plane) → staphylo (grapelike clusters)



 Staphylococci
 Image: Constraint of the second s

This is why the shape of the bacteria is very important in <u>identification</u> and <u>naming</u> of bacteria. For example staphylococcus (staphylo: grape-like clusters, coccus: spherical). It is an <u>opportunistic</u> bacteria, which means it becomes pathogenic under certain circumstances **ONLY**.

26:00-37:00

Bacterial Cell Structure

Bacterial cell structure is much simpler than eukaryotic cells. If you put a bacterial cell under the TEM (Transmission Electron Microscope) you can notice how simple the structure is. You can see:

- 1. Bacterial cell wall: which is the outermost cell layer
- 2. Plasma membrane: beneath the cell wall.

Both the cell wall and the plasma membrane make what we call the <u>CELL</u> <u>ENVELOPE.</u>

- 3. Nucleoid: A dark area which contains DNA. It is not a true nucleus
- 4. Ribosomes: Very small organelles

5. **Cytoplasm**: (cytosol) a fluid that contains dissolved materials, such as enzymes, proteins, lipids, polysaccharides, electrolytes

Prokaryotes in general have higher concentration of dissolved solutes in their cytoplasm than eukaryotes, because they don't have membrane bound organelles. In eukaryotic cells, these dissolved minerals are distributed among the cytoplasm and among organelles, while in prokaryotes, they are mainly found in the cytoplasm, giving a thick gel-like cytoplasm and a high osmotic pressure.

What actually stops the bacterial cells from bursting in the human fluid is the bacterial cell wall. Human fluid is considered hypotonic to bacterial cells, since the osmotic pressure for a bacterial cell is much higher than a human cell. Bacterial cells would burst by osmosis if there was no cell wall, so the cell wall maintains a <u>high cell turgidity.</u>

37:00-40:00

Always believe in yourself, don't let anything in life stand between you and your goals ...GOOD LUCK ©